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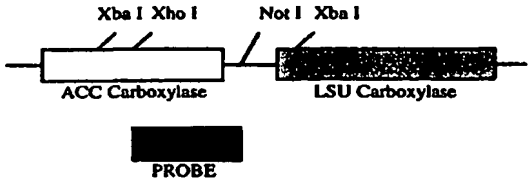

(57) Abstract

Novel compositions and methods useful for genetic engineering of plant cells to provide expression of cellulose degrading enzymes in the plants or plant cells.

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EXPRESSION OF ENZYMES INVOLVED IN CELLULOSE MODIFICATION

INTRODUCTION

This application is a continuation-in-part of Application Serial No. 09/122,533 filed July 24, 1998.

Technical Field

This invention relates to the application of genetic engineering techniques to plants. More specifically, the invention relates to compositions and methods for expressing polysaccharide hydrolyzing enzymes (cellulases, cellobiohydrolases, xylanases, hemicellulases) in plant plastids.

Background

Polysaccharide hydrolyzing enzymes are a family of enzymes that work together to break down cellulose to its simple sugar components. Cellulose may also be degraded industrially via acid hydrolysis using much harsher conditions than required by enzymes. Furthermore, polysaccharide hydrolyzing enzymes catalyze highly specific reactions, yielding specific products, and are required in much smaller quantities compared to acid hydrolysis reactions.

Cellulose degrading enzymes are used for a wide variety of industrial applications. One of the major potential uses of such enzymes is in the conversion of cellulosic biomass to industrially important end products (for example, sugars, which can be fermented to produce a variety of products). For example, in the production of fuel, ethanol can be produced from grains such as corn. A similar process utilizing high cellulosic rice straws is currently under development. Unfortunately, ethanol produced by such methods is still too expensive to compete commercially with gasoline. However, improvements in technology to utilize wood, grass and other high cellulose containing biomass for the production of ethanol would be valuable to the art for production of a less expensive and cleaner fuel source.

In addition to biomass conversion, cellulose degrading enzymes find utility in a variety of other industrial products and processes including: textile finishing, production of detergent additives, food and beverage processing, feed additives, ensiling and fermentation processes.

Current methods for the production of cellulose degrading enzymes are generally believed to be limiting to the further development of a lignocellulosic ethanol industry. Filamentous fungi are well known for the production of industrial cellulases. However, economical production of cellulose degrading enzymes is compounded by the relatively slow growth rates of cellulose degrading enzyme producing fungi, the long times required for enzyme induction an the high value of the product ethanol.

Recently, genes encoding cellulose degrading enzymes have been cloned from a variety of cellulytic bacteria and fungi. Cloned genes encoding cellulases having very high specific activities over a broad pH range in addition to high thermostability are considered most desirable for bioethanol derived processes.

Recombinant bacterial or fungal hosts producing cellulose degrading enzymes have been the focus of recent efforts for the production of various cellulase preparations. However, methods for the production of polysaccharide degrading enzymes in plant cells are needed. Such methods would provide a less expensive and abundant source of cellulose degrading enzymes.

SUMMARY OF THE INVENTION

The present invention provides methods and compositions for the production of polysaccharide hydrolyzing enzymes in plant cells. The methods generally involve the use of an expression construct having a promoter, a nucleic acid sequence encoding a polysaccharide hydrolyzing enzyme, and a transcription termination region for plant transformation. Preferably, the promoter is functional in a plant cell plastid.

By this invention, a method is disclosed whereby constructs encoding a polysaccharide hydrolyzing enzyme can be produced in plant cells. In one embodiment of the present invention, methods are disclosed for the high level expression of a polysaccharide hydrolyzing enzyme in plant plastids.

The method provided for high level expression of polysaccharide hydrolyzing enzyme in plant cells offers a novel means for the production of cellulose degrading enzymes (cellulases, cellobiohydrolases, xylanases, hemicellulases) in plant cells. The method generally comprises growing a plant having integrated into its genome a construct comprising as operably linked components in the 5' to 3' direction of transcription, a transcription initiation region functional in a plant cell, a DNA sequence encoding a polysaccharide hydrolyzing enzyme and a transcription termination sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the primary DNA vector pCGN6115 for plant plastid transformation. Figure 1 also shows a schematic representation of the site of homologous recombination in the plastid genome. The upper line represents the transgene encoding the E1 cellulase, aadA (strep/spec) marker gene for selection of plastid transformants and plastid homology sequences. The middle line represents the region of the chloroplast genome for integration of the transgene, and the bottom line represents the nucleic acid probe used in Southern hybridization for determination of plastid transformants.

Figure 2 shows the result of the Southern hybridization utilizing the probe depicted. The wild type band is in lane 1. A plasmid (pCGN6115)control is in lane 3. Several subclones of the same transgenic events were analyzed on the same blot. Each subclone was regenerated from the same inital transformant. For example, in event 6115-14, there are 4 subclones of the same event, all are homplasmic for the inserted genes. Some subclones are wild type in the other events.

Figure 3 shows the results of Western hybridization of total soluble leaf protein extracted from homoplasmic tobacco lines transformed with the pCGN6115 construct using monoclonal antibodies raised to purified *Acidothermus cellulolyticus* E1 β-1,4-endoglucanase.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, plastid expression constructs are provided which generally comprise a promoter functional in a plant cell, a DNA sequence encoding a polysaccharide hydrolyzing enzyme and a transcription termination region capable of

terminating transcription in a plant cell. These elements are provided as operably joined components in the 5' to 3' direction of transcription.

The polysaccharide hydrolyzing enzyme of the present invention can be obtained from any source. The polysaccharide hydrolyzing enzyme of the present invention is preferably obtained from a non-plant source. Preferably, the polysaccharide hydrolyzing enzyme is a cellulase, hemicellulase, and ligninase.

Polysaccharide hydrolyzing enzymes are a family of enzymes which break down cellulose to its simple sugar components. The family includes several classes of enzymes, including but not limited to cellulase, hemicellulase, and ligninase.

Polysaccharides, as used herein, refer to polymers of more than about ten monosaccharide residues linked glycosidically in branched or unbranched chains. A monosaccharide refers to a simple sugar which cannot be hydrolyzed to smaller units. The empirical formula is $(CH_2O)_n$ and range in size from trioses (n=3) to heptoses (n=7).

A cellulase enzyme, as used herein, includes, but is not limited to endocellulases and exocellulases. Endocellulases, for example, include enzymes such as E1 cellulase from *Acidothermus cellulolyticus* (described in U.S. Patent Number 5,536,655). Exocellulases, for example, include enzymes such as cellobiohydrolase 1 (CBH1) from *Trichoderma reesei* (Shoemaker, *et al.* (1983) *Biotechnology*, 1:691-696).

A hemicellulase, as used herein includes, but is not limited to xylanase. Nucleic acid sequences encoding xylanases are known in the art, and include, *xyn*A from *Acidobacterium capsulatum* (Inagaki, *et al.* (1998) *Biosci. Biotechnol. Biochem.* 62(6):1061-1067) and *xyn*A from *Clostridium thermocellum* (Hayashi, *et al.* (1999), *Appl. Microbiol. Biotech.*, 51(3):348-357)

A ligninase, as used herein, includes, but is not limited to, laccase. Nucleic acid sequences for use in the methods of the present invention include, but are not limited to *lac*AL from *Schizophyllum commune* (Hatamoto, *et al.* (1999) *Biosci. Biotechnol. Biochem.*, 63(1):58-64), *lcc*1 from *Pichia pastoris* (Jonsson, *et al.* (1997) *Curr Genet*, 32(6):425-530) and *lcc*I and *lcc*IV from *Trametes versicolor* (Ong, *et. al* (1997) *Gene* 196(1-2):113-119).

In one aspect of the present invention, expression constructs are prepared to direct the expression of polysaccharide hydrolyzing enzymes in a plant cell. The constructs generally contain a promoter functional in a plant cell, a nucleic acid sequence encoding polysaccharide hydrolyzing enzyme, and a transcription termination region functional in a plant cell.

Promoters for use in the constructs of the present invention include promoters functional in a plant cell nucleus, or promoters functional in plant cell organelles, such as mitochondria and plastids. Those skilled in the art will recognize that there are a number of promoters which are functional in plant cells, and have been described in the literature. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned.

One set of promoters are constitutive promoters such as the CaMV35S or FMV35S promoters that yield high levels of expression in most plant organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are useful in the practice of this invention (Odell, *et al.* (1985) *Nature* 313:810-812; Rogers, U.S. Patent Number 5,378, 619). In addition, it may also be preferred to bring about expression of the cellulose degrading enzyme in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity.

It may be advantageous to direct the localization of the polysaccharide degrading enzymes to a particular subcellular compartment, for example, to the mitochondrion, endoplasmic reticulum, vacuoles, chloroplast or other plastidic compartment. For example, where the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression, the constructs will also employ the use of sequences to direct the gene to the plastid. Such sequences are referred to herein as chloroplast transit peptides (CTP) or plastid transit peptides (PTP). In this manner, where the gene of interest is not directly inserted into the plastid, the expression construct will additionally contain a gene encoding a transit peptide to direct the gene of interest to the plastid. The chloroplast transit peptides may be derived from the gene of interest, or may be derived from a heterologous sequence having a CTP. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and, Shah *et al.* (1986) *Science 233*:478-481. Additional transit peptides for the translocation of the protein to the endoplasmic reticulum (ER), or vacuole may also find use in the constructs of the present invention.

Preferably, the promoter employed in the constructs of the present invention are functional in a plant cell plastid. A number of promoters functional in plastids are known in the art, and include, but are not limited to the 16S rRNA promoter region, *Prrn* (Svab, *et al.*

(1993), *Proc. Natl. Acad. Sci. USA*, 90:913-917) and the D1 thylakoid membrane protein promoter region, P*psb*A (Staub, *et al.*, (1993), *EMBO J.*, 12(2):601-606).

Preferably, the transcription termination region employed in the constructs of the present invention are functional in a plant cell plastid. A number of such termination regions are available in the art, and include, but are not limited to the D1 thylakoid membrane protein termination region, T*psb*A (Staub, *et al.*, (1993), *EMBO J.*, 12(2):601-606).

In the examples described herein, nucleic acid sequences encoding a thermophilic E1 cellulase from *Acidothermus cellulolyticus*, as well as sequences encoding cellobiohydrolase I (CBH1), laccase, and xylanase are employed in constructs to direct expression from the plastid of plant cells. Furthermore, transplastomic tobacco plants expressing E1 cellulase demonstrate a high level of expression of the cellulase enzyme.

In addition, the expressed enzyme demonstrates similar enzymatic characteristics as the extracted wild type enzyme. For example, in the examples provided below, crude protein extracts containing the expressed cellulase from homoplasmic tobacco plants exhibits a higher activity at 80°C than at 55°C. Thus, a thermophilic cellulase expressed from the plant plastid as described in the instant invention exhibits the same thermophilic properties as the wild type cellulase which has a temperature optimum of 83°C (described in USPN 5,536,655, the entirety of which is incorporated herein by reference). A thermophilic cellulase with increased activity above 45°C provides an safeguard against cellulase activity during cultivation of the transformed plant in a production field. Preferred would be cellulases with activities optimized about or above 55°C.

An artisan skilled in the art to which the present invention pertains will recognize that enzymes from other sources may be utilized in plastid expression constructs of the present invention. For example, DNA sequences encoding for other polysaccharide hydrolyzing enzymes such as those from *Thermomonospora fusca* (*See, e.g.*, Wilson (1992) *Crit. Rev. Biotechnol.* 12:45-63) may be used in the expression constructs of the present invention.

The constructs of the present invention may be integrated into the host plant cells nuclear genome, and the enzyme is targeted to a cellular organelle. For example, sequences directing the expressed enzyme to the vacuole may be employed, as well as sequences directing the transport to the plastid. Such plastid transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep. 9*:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and, Shah *et al.* (1986)

Science 233:478-481. The protein signal for targeting to vacuoles can be provided from a plant gene which is normally transported across the rough endoplasmic reticulum, such as the 32 amino acid N-terminal region of the metallocarboxypeptidase inhibitor gene from tomato (Martineau *et al.* (1991) *Mol. Gen. Genet. 228* :281-286). In addition to the signal sequence, vacuolar targeting constructs also encode a vacuolar localization signal (VLS) positioned at the carboxy terminus of the encoded protein. Appropriate signal sequences and VLS regions can be obtained from various other plant genes and can be similarly used in the constructs of this invention. Numerous vacuolar targetting peptides are known to the art, as are reviewed in Chrispeels *et al.*, Cell (1992) 68:613-616.

In developing the constructs the various fragments comprising the regulatory regions and open reading frame may be subjected to different processing conditions, such as ligation, restriction enzyme digestion, PCR, *in vitro* mutagenesis for improved enzymes, linkers and adapters addition, and the like. Thus, nucleotide transitions, transversions, insertions, deletions, or the like, can be performed on the DNA which is employed in the regulatory regions or the DNA sequences of interest for expression in the plastids. Methods for restriction digests, Klenow blunt end treatments, ligations, and the like are well known to those in the art and are described, for example, by Maniatis *et al.* (in *Molecular Cloning: A Laboratory Manual* (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

During the preparation of the constructs, the various fragments of DNA will often be cloned in an appropriate cloning vector, which allows for amplification of the DNA, modification of the DNA or manipulation of the DNA by joining or removing sequences, linkers, or the like. Preferably, the vectors will be capable of replication to at least a relatively high copy number in *E. coli*. A number of vectors are readily available for cloning, including such vectors as pBR322, vectors of the pUC series, the M13 series vectors, and pBluescript vectors (Stratagene; La Jolla, CA).

In order to provide a means of selecting the desired plant cells, vectors for plastid transformation typically contain a construct which provides for expression of a selectable marker gene. Marker genes are plant-expressible DNA sequences which express a polypeptide which overcomes a natural inhibition by, attenuates, or inactivates a selective substance, *i.e.*, antibiotic, herbicide *etc.*.

Alternatively, a marker gene may provide some other visibly reactive response, *i.e.*, may cause a distinctive appearance or growth pattern relative to plants or plant cells not

expressing the selectable marker gene in the presence of some substance, either as applied directly to the plant or plant cells or as present in the plant or plant cell growth media.

In either case, the plants or plant cells containing such selectable marker genes will have a distinctive phenotype for purposes of identification, *i.e.*, they will be distinguishable from non-transformed cells. The characteristic phenotype allows the identification of cells, cell groups, tissues, organs, plant parts or whole plants containing the construct.

Detection of the marker phenotype makes possible the selection of cells having a second gene to which the marker gene has been physically linked. This second gene typically comprises a desirable phenotype which is not readily identifiable in transformed cells, but which is present when the plant cell or derivative thereof is grown to maturity, even under conditions wherein the selectable marker phenotype itself is not apparent.

The use of such a marker for identification of plant cells containing a plastid construct has been described by Svab *et al.* (1993, *supra).* In the examples provided below, a bacterial *aad*A gene is expressed as the marker under the regulatory control of chloroplast 5' promoter and 3' transcription termination regions, specifically the regulatory regions of the*psb*A gene (described in Staub *et al.*, (1993), *supra*). Numerous additional promoter regions can also be used to drive expression of the selectable marker gene, including various plastid promoters and bacterial promoters which have been shown to function in plant plastids.

Expression of the *aad*A gene confers resistance to spectinomycin and streptomycin, and thus allows for the identification of plant cells expressing this marker. The *aad*A gene product allows for continued growth and greening of cells whose chloroplasts produce the selectable marker gene product. Cells which do not contain the selectable marker gene product are bleached. Selection for the *aad*A gene marker is thus based on identification of plant cells which are not bleached by the presence of streptomycin, or more preferably spectinomycin, in the plant growth medium.

A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. Other genes which encode a product involved in chloroplast metabolism can also be used as selectable markers. For example, genes which provide resistance to plant herbicides such as glyphosate, bromoxynil or imidazolinone may find particular use. Such genes have been reported (Stalker *et al., J. Biol. Chem.* (1985) *260*:4724-4728 (glyphosate resistant EPSP); Stalker *et al., J. Biol. Chem.* (1985) *263*:6310-6314 (bromoxynil resistant nitrilase gene); and Sathasivan *et al., Nucl. Acids Res.* (1990) *18*:2188 (AHAS imidazolinone resistance gene)).

Methods of plant nuclear transformation and selection which employ a biolistic, or bombardment, method to transfer the target DNA constructs to plant cells can also be used in the instant invention. Such methods are particularly useful in transformation of plant cells which are less susceptible to *Agrobacterium*-mediated transformation methods. Bombardment tranformation methods are described in Sanford *et al.* (1991) *Technique 3*:3-16; Klein *et al.* (1992) *Bio/Technology 10*:286-291

Generally in transformation of plant cells, target explants are incubated with the transformed *Agrobacterium*, for example as described by Horsch *et al.* (*Science* (1985) *227*:1229-1232), or bombarded with DNA coated particles. The plant cells are then grown in an appropriate medium to selectively culture those plant cells which have obtained the desired constructs. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants can then be grown and either pollinated with the same transformed strain or different strains. For production of a homozygous line, self pollination is used.

Stable transformation of tobacco plastid genomes by particle bombardment is reported (Svab *et.al.* (1990), *supra*) and Svab *et al.* (1993), *supra*). The methods described therein may be employed to obtain plants homoplasmic for plastid expression constructs.

Generally, bombarded tissue is cultured for approximately two days on a cell division-promoting media, after which the plant tissue is transferred to a selective media containing an inhibitory amount of the particular selective agent, as well as the particular hormones and other substances necessary to obtain regeneration for that particular plant species. Shoots are then subcultured on the same selective media to ensure production and selection of homoplasmic shoots.

Homoplasmy is verified by Southern analysis for plants transplastomic for the gene encoding the protein of interest. In the examples provided below, *Xba* I-digested total cellular DNA is tested with a radio labelled probe, specifically, a part of the plastid targeting fragment, including the *aad*A marker gene, and sequence of the integration region containing the acetyl CoA carboxylase DNA sequence. Southern blot analysis with this probe confirms the integration of the chimeric E1 cellulase gene in the tobacco plastid genome to yield transplastome lines.

Where transformation and regeneration methods have been adapted for a given plant species, either by *Agrobacterium*-mediated transformation, bombardment or some other

method, the established techniques may be modified for use in selection and regeneration methods to produce plastid-transformed plants. For example, the methods described herein for tobacco are readily adaptable to other solanaceous species, such as tomato, petunia and potato.

For transformation of soybean and other plant species, particle bombardment as well as *Agrobacterium*-mediated nuclear transformation and regeneration protocols have been described (Hinchee *et al.* USPN 5,416,011, and Christou *et al.* USPN 5,015,580). The skilled artisan will recognize that protocols described for soybean transformation may be used and adapted to other plant species.

In *Brassica*, *Agrobacterium*-mediated transformation and regeneration protocols generally involve the use of hypocotyl tissue, a non-green tissue which might contain a low plastid content. Thus, for *Brassica*, preferred target tissues would include microspore-derived hypocotyl or cotyledonary tissues (which are green and thus contain numerous plastids) or leaf tissue explants. While the regeneration rates from such tissues may be low, positional effects, such as seen with *Agrobacterium*-mediated transformation, are not expected, thus it would not be necessary to screen numerous successfully transformed plants in order to obtain a desired phenotype.

For cotton, transformation of *Gossypium hirsutum* L. cotyledons by co-cultivation with *Agrobacterium tumefaciens* has been described by Firoozabady *et al.*, *Plant Mol. Bio.* (1987) *10*:105-116 and Umbeck *et al.*, *Bio/Technology* (1987) *5*:263-266. Again, as for *Brassica*, this tissue may contain insufficient plastid content for chloroplast transformation. Thus, as for *Brassica*, an alternative method for transformation and regeneration of alternative target tissue containing chloroplasts may be desirable, for instance targeting green embryogenic tissue.

Other plant species may be similarly transformed using related techniques. Alternatively, microprojectile bombardment methods, such as described by Klein *et al.* (*Bio/Technology 10*:286-291) may also be used to obtain nuclear transformed plants comprising the viral single subunit RNA polymerase expression constructs described herein. Cotton transformation by particle bombardment is reported in WO 92/15675, published September 17, 1992. Plants for the practice of the present invention include, but are not limited to, soybean, cotton, alfalfa, oil seed rape, flax, tomato, sugar beet, sunflower, potato, tobacco, *maize*, wheat, rice and lettuce.

Methods for the transformation of corn, wheat and rice are well known in the art, and are described for example in U.S. Patent Numbers 5,538,877 and 5,538,880, U.S. Patent Numbers 5,610,042, and European Patent Number EP 539563.

The vectors for use in plastid transformation preferably include means for providing a stable transfer of the plastid expression construct and selectable marker construct into the plastid genome. This is most conveniently provided by regions of homology to the target plastid genome. The regions of homology flank the construct to be transferred and provide for transfer to the plastid genome by homologous recombination, via a double crossover into the genome. The complete DNA sequence of the plastid genome of tobacco has been reported (Shinozaki *et al.*, *EMBO J.* (1986) *5*:2043-2049). Complete DNA sequences of the plastid genomes from liverwort (Ohyama *et al.*, *Nature* (1986) *322*:572-574) and rice (Hiratsuka *et al.*, *Mol. Gen. Genet.* (1989) *217*:185-194), have also been reported.

Where the regions of homology are present in the inverted repeat regions of the plastid genome (known as IRA and IRB), two copies of the transgene are expected per transformed plastid. The regions of homology within the plastid genome are approximately 1kb in size. Smaller regions of homology can also be used, and as little as 100 bp can provide for homologous recombination into the plastid genome. However, the frequency of recombination and thus the frequency of obtaining plants having transformed plastids decreases with decreasing size of the homology regions.

Examples of constructs having regions of homology from the plastid genome are described in Svab *et.al.* (1990 *supra*), Svab *et al.* (1993 *supra*) and Zoubenko *et al.* (*Nuc Acid Res* (1994) 22(19):3819-3824). In the examples provided herein, the flanking tobacco plastid homology regions of the plastid expression construct direct the insertion of an E1 cellulase transgene into the tobacco chloroplast genome between acetyl CoA carboxylase (ORF512) and the large subunit of RuBisCo (*rbc*L). Such regions of homology are described in Svab and Maliga (1993) *supra*. Since integration into the plastid genome occurs by homologous recombination and the target site is not in an inverted repeat region of the plastid genome, one copy of the transgene per plastid genome is expected. Selection is made for the spectinomycin resistance marker phenotype expressed by the *aad*A gene.

Since one of the major components of plants is cellulose, it would be expected that the production of cellulose degrading enzymes in plants cells may have detrimental effects to the host organism. However, by compartmentalizing the expressed cellulose degrading enzyme in a plant organelle, for example in a plastid, any detrimental effects of cellulase enzyme

expression may be overcome. Furthermore, utilization of a cellulose degrading enzyme with a high temperature and/or pH optimum may also provide safeguards for the expression of such enzymes in plants that are grown at ambient temperatures.

Plant plastids (chloroplasts, amyloplasts, elaioplasts, chromoplasts, etc.) are the major biosynthetic centers that, in addition to photosynthesis, are responsible for production of industrially important compounds such as amino acids, complex carbohydrates, fatty acids, and pigments. Plastids are derived from a common precursor known as a proplastid and thus the plastids present in a given plant species all have the same genetic content. Plant cells contain 500-10,000 copies of a small 120-160 kilobase circular genome, each molecule of which has a large (approximately 25kb) inverted repeat. Thus, it is possible to engineer plant cells to contain up to 20,000 copies of a particular gene of interest which potentially can result in very high levels of foreign gene expression. In addition, plastids of most plants are maternally inherited. Consequently, heterologous genes expressed in plastids are not pollen disseminated, therefore, a trait introduced into a plant plastid will not be transmitted to wild-type relatives by cross-fertilization. Thus, the plastids of higher plants are an attractive target for genetic engineering.

The present invention also provides methods for the production of heat stable industrial enzymes in a host plant cell. Such methods generally include the use of the expression constructs of the present invention in the production of transgenic plants. Preferably, the methods involve the expression of a thermostable polysaccharide degrading enzyme in a plant cell, growing a plant having transformed cells, collecting plants or plant parts, and subjecting the collected plant material to conditions which promote optimal enzyme activity.

The present invention further provides methods for encapsulating cell wall degrading enzymes in organelles, such as vacuoles, plastids, and the like. The methods generally involve expressing polysaccharide degrading enzyme in a plant cell nucleus, and localizing the enzyme in a plant cell organelle using a organellular targeting sequence. Alternatively, the enzyme can be expressed directly from a plant cell organelle, for example a plant cell plastid.

The methods of the present invention can also include additional sequences and constructs involved in the modification of polysaccharides in plant cells. Such sequences and constructs are known in the art and include, but are not limited to those described in PCT Publication WO 98/18949, the entirety of which is incorporated herein by reference.

The methods of the present invention can also include the expression of one or more expression constructs providing for the expression of one or more sequences encoding polysaccharide degrading enzymes. For example, one expression cassette can be introduced into a plant cell providing for the expression of an endocelluase, and a second expression cassette can also be introduced for the expression of a ligninase.

Thus, the present invention provides novel methods for the production of thermophilic polysaccharide hydrolyzing enzymes (i.e., cellobiohydrolase, xylanase, hemicellulase) in plant cells. Expression of such enzymes in plant cells provides an alternative source for the production of polysaccharide degrading enzymes utilized for industrial products/processes (such as textile finishing, detergents, food and beverage processing, feed additives, ensiling, pulping, paper making, and biomass conversions). Also, the expression of thermophilic cellulases and related cellulose degrading enzymes in plant plastids provides an alternative or supplementary method for degrading endogenous cellulose contained in plant tissues and releasing the stored carbon (as monosaccharides) for subsequent fermentative processes. In addition, enzyme sequestration (isolation from the substrate) and/or the high temperature optimum (versus low activity at ambient temperatures)of the thermophilic cellulase provide two internal safeguards for protecting the plant from the intrinsic enzyme activity during critical plant growth and development stages.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1 Construct Preparation

Constructs and methods for use in transforming the plastids of higher plants are described in Zoubenko *et al.* (*Nuc Acid Res* (1994) 22(19):3819-3824), Svab *et al.* (*Proc. Natl. Acad. Sci.*(1990) 87:8526-8530 and *Proc. Natl. Acad. Sci.*(1993) 90:913-917) and Staub *et al.* (*EMBO J.* (1993) 12:601-606). The complete DNA sequences of the plastid genome of tobacco are reported by Shinozaki *et al.* (*EMBO J.* (1986) 5:2043-2049). All plastid DNA references in the following description are to the nucleotide number from tobacco.

A vector was prepared to direct the expression of the *Acidothermus* E1 β-1,4-endoglucanase in plant plastids. The plasmid pMPT4, a pGEM (Clonetech) derivative containing the entire *Acidothermus* E1 cellulase coding sequence (U.S. Patent Number

5,536,655) and flanking regions on a 3.7 kb *Pvu* I genomic DNA fragment, was digested with the restriction endonuclease sites *Sac*II and *Asp*718 to remove the coding sequence for the mature E1 cellulase protein. This fragment was cloned into the same restriction sites of the plasmid pBCSK+ (Stratagene)to create the vector pCGN6063. This plasmid was digested with *Sac*I and *Sac*II and a double-stranded oligonucleotide sequence, 5'-GGAGCTGCGTCACCATGGCGGGA-3', was inserted to introduce an *Nco*I site-derived ATG translational start codon fused to the 60,000 mol wt mature sequence (minus the endogeneous bacterial signal peptide amino acid sequence) of the E1 β-1,4-endoglucanase polylpeptide, creating the construct pCGN6067. The E1 gene was excised from pCGN6067 as an *Nco* I to *Asc* I DNA segment and cloned into the T7 promoter expression cassette pCGN5063 to create the construct pCGN6108. This plasmid contains the plastid expression regulatory elements of the T7 bacteriophage promoter operably linked to the mature protein portion of the E1 coding sequence and *psb*A transcription termination region. The chimeric cellulase expression cassette was excised as a *Hind* III to *Not* I DNA fragment and cloned into the tobacco chloroplast homology vector, pCGN6043 in the same restriction sites to create the construct pCGN6115. The homology sequences employed in the vector direct the integration of the E1 cellulase gene and aadA marker transgene to the region between the *rbc*L and ORF512 sequences (described in Svab *et al.*, (1993) *supra*). The construct pCGN6115 (Figure 1) was used to transform tobacco plants to direct the transformation to homoplasmy and plastid expression of the E1 β-1,4-endoglucanase encoding gene in the plant plastid.

In order to express the nucleic acid sequence encoding xylanase from *Dictyoglomus thermophilum* (Gibbs, *et al.* (1995) *Appl. Environ. Microbiology*, 61(12):4403-4408) oligonucleotide primers can be synthesized for use in Polymerase Chain Reactions to amplify the entire coding region of the *Xyn*A gene. The two primers, 5'-ATGGTACCATGCTTAACCAAAGGTTTTCTATC (the underlined sequence represents the initiation ATG) containing a *Nco*I site at the 5' terminus for cloning into the T7 promoter, and 5'-CCTATAGGCGCGCCAAAACTTTACAATCTCCC containing a *Asc*I site at the 5' end for cloning, can be used to amplify the entire coding region of the *Xyn*A sequence. The 1300 base pair amplification product can then be cloned into the T7 promoter expression cassette pCGN5063 to create the construct pCGN6581. This plasmid contains the plastid expression regulatory elements of the T7 bacteriophage promoter operably linked to the mature protein portion of the *Xyn*A coding sequence and *psb*A transcription termination region. The

chimeric xylanase expression cassette can be excised as a *Hind* III to *Not* I DNA fragment and cloned into the tobacco chloroplast homology vector, pCGN6043 in the same restriction sites to create the plastid transformation construct pCGN6582.

In order to express the nucleic acid sequence encoding CBH1 from *Trichoderma reesei* (Shoemaker, *et al.* (1983) *Biotechnology*, 1:691-696) oligonucleotide primers can be synthesized for use in Polymerase Chain Reactions to amplify the entire coding region of the CBH1 gene. The two primers, 5'-TGGCACCATGCATCGGAAGTTGGCCGTCA (the underlined sequence represents the initiation ATG) containing a *Nco*I site at the 5' terminus for cloning into the T7 promoter, and 5'-CCTATAGGCGCGCCCAGGCACTGAGAGTAGTAAGG containing a *Asc*I site at the 5' end for cloning, can be used to amplify the entire coding region of the CBH1 sequence. The approximately 1700 base pair amplification product can then be cloned into the T7 promoter expression cassette pCGN5063 to create the construct pCGN6583. This plasmid contains the plastid expression regulatory elements of the T7 bacteriophage promoter operably linked to the mature protein portion of the CBH1 coding sequence and *psb*A transcription termination region. The chimeric CBH1 expression cassette can be excised as a *Hind* III to *Not* I DNA fragment and cloned into the tobacco chloroplast homology vector, pCGN6043 in the same restriction sites to create the plastid transformation construct pCGN6584.

In order to express the nucleic acid sequence encoding laccaseI from *Aspergillus nidulans* (Aramayo, *et al.* (1990) *Nucleic Acids Research*, 18(11):3415) oligonucleotide primers can be synthesized for use in Polymerase Chain Reactions to amplify the entire coding region of the *yA* gene. The two primers, 5'-ATCCAGCCATGCACCTCTCCACGGTCCTCTTCCA (the underlined sequence represents the initiation ATG) containing a *Nco*I site at the 5' terminus for cloning into the T7 promoter, and 5'-TATGAGGGCGCGCCCTAAGAATCCCAAACATCAACCCCG containing a *Asc*I site at the 5' end for cloning, can be used to amplify the entire coding region of the *yA* sequence. The approximately 2100 base pair amplification product can then be cloned into the T7 promoter expression cassette pCGN5063 to create the construct pCGN6585. This plasmid contains the plastid expression regulatory elements of the T7 bacteriophage promoter operably linked to the mature protein portion of the *yA* coding sequence and *psb*A transcription termination region. The chimeric laccase expression cassette can be excised as a *Hind* III to *Not* I DNA fragment and cloned into the tobacco chloroplast homology vector,

pCGN6043 in the same restriction sites to create the plastid transformation construct pCGN6586.

Example 2 Plant Plastid Transformation

Tobacco plants transformed to express T7 polymerase from the nuclear genome and targeted to the plant plastid are obtained as described in McBride et al U.S. Patent Number 5,576,198. Transgenic tobacco plants homozygous for the plastid targeted T7 polymerase are used for plastid transformation using particle bombardment.

Tobacco plastids are transformed by particle gun delivery of microprojectiles. Since integration into the plastid genome occurs by homologous recombination and the target site is between the acetyl CoA carboxylase and the large subunit of RuBisCo (*rbc*L), a single copy of the transgene is expected per plastid genome (Svab *et al.* (1993) *supra*).

Tobacco seeds (N. *tabacum* v. Xanthi N/C) homozygous for pCGN4026 (McBride *et al.*, U.S. Patent Number 5,576,198) T-DNA are surface sterilized in a 50% chlorox solution (2.5% sodium hypochlorite) for 20 minutes and rinsed 4 times in sterile H_2O. The seeds are then plated asceptically on a 0.2X MS salts media and allowed to germinate. The seedlings are grown on agar solidified MS media with 30g/l sucrose (Murashige and Skoog (1962) *Physiol. Plant 15*:493-497).

Tungsten microprojectiles (1.0μm) are coated with DNA, such as the T7/E1 cellulase expression construct, pCGN6115, and the coated microprojectiles used to bombard mature leaves, placed abaxial side up on RMOP media (MS salts, 1 mg/l BAP, 0.1 mg/l NAA, 30 g/l sucrose and 0.7% phytager) (Svab *et al.* (1990) *supra*) using the Bio-Rad PDS 1000/He bombardment system (Sanford *et al.* (1991) *Technique 3*:3-16; Klein *et al.* (1992) *Bio/Technology 10*:286-291). Development of transformed plants on RMOP media supplemented with 500 mg/l spectinomycin dihydrochloride and subsequent subcloning on the same selective medium is conducted according to Svab *et al.* (1990); Svab and Maliga (1993); *supra*). Selected plants are rooted in MS media containing 1 mg/l IBA, 500 mg/l spectinomycin dihydrochloride and 0.6% phytagar.

Example 3 Analysis of Cellulase Expression in Plastids

Following plastid transformation as described above, five independently isolated homoplasmic lines generated in the nuclear encoded T7 RNA polymerase producing background were generated. A schematic of pCGN6115 construct and a representation of

incorporation into the tobacco plastid genome is shown in Figure 1. The upper line represents the incoming DNA donated from pCGN6115 and the middle line represents the integration target region. Expected sizes for *Xba*I fragments are shown for the incoming DNA as well as for wild type DNA. As there is no *Xba*I site on the 5' end of the incoming DNA the combined size of the two chimeric genes is indicated. Also shown in Figure 1 is the location of the probe used for Southern analysis. Homoplasmy was determined by Southern blot analysis as shown in Figure 2.

To confirm homoplasmy by Southern hybridization, total plant cellular DNA is prepared as described by Bernatzky and Tanksley ((1986) *Theor Appl Genet.* 72:314-321). Approximately 3 μg DNA for each sample is digested with *Xba*I, electrophoresed through 0.7% agarose, transferred to Nytran+ (Schleicher and Schuell). The filters were hybridized in buffer (50% formamide, 6x SSC, 5X Denhardt's solution, 0.5% SDS, 150μg/ml Salmon sperm DNA) at 42°C with alpha ^{32}P-dCTP labeled probe. The hybridization probe was prepared from a nucleic acid sequences spanning the the integration zone. This DNA sequence contains approximately 50% of the native chloroplast gene acetyl CoA Carboxylase and some flanking intergenic sequence, and is derived as a NotI/XhoI fragment from pCCN6042, a precursor plasmid containing only the tobacco plastid homology sequences present in plasmid pCGN6115.

The results of the Southern hybridization are shown in Figure 2. Homoplasmic lines are identified which contain the E1 cellulase and *aad*A coding sequences. Non-transformed control tobacco lines (wild-type Xanthi) probed with the 6042 DNA fragment hybridize with a 1.5 kb DNA fragment, while homoplasmic tobacco lines containing the E1 cellulase and *aad*A sequences hybridize with a 2.5 kb DNA fragment. Transplastomic lines which are heteroplasmic demonstrate a hybridization pattern containing both the wild-type DNA fragment, 1.5 kb, and the homoplasmic DNA fragment, 2.5 kb. The difference in band size between the transgenic plants and wild type is the presence of the aadA resistance gene and its regulatory sequences in the transgenic plants. This adds approximately 1 kb to the wild type band.

To demonstrate that the homoplasmic 6115 tobacco lines express the E1 β-1,4-endoglucanase, Western blot analysis was performed using total soluble leaf protein. Leaf protein was extracted as follows: 200mg mature leaf samples were frozen in liquid N2 and ground in 0.08 ml extraction buffer containing 0.1M $NaPO_4$ pH6.8, 0.15M NaCl, 0.01M EDTA, 0.01M DTT, 0.01M thiourea, 0.3% Tween-20, 0.05% Triton-X100. Protein

concentrations were determined by Bradford assay. Protein samples were combined with an equal volume of 2x Laemmli sample buffer (Laemmli (1970) *Nature* 227:680-685) and boiled prior to loading onto 10% Laemmli gels. Approximately 40ug of total leaf protein was loaded/lane.

Results of the Western blot analysis (Figure 3) using monoclonal antibodies raised against the *Acidothermus* E1 cellulase demonstrate that the E1 protein is expressed in all homoplasmic 6115 lines examined. 250 ng of E1 cellulase purified from *Strepromyces* was loaded onto the first lane. This protein runs as multiple forms on a denaturing gel, the highest form being 72,000 molecular weight as this form includes the signal peptide for secretion. The mature form of the enzyme is around 60 kd. The second lane contains control tobacco tissue. The third lane contains an extract from a 6115 homoplasmic plant that does not contain the T7 RNA polymerase to activate E1 cellulase expression in the plastid. Lanes 4-8 are independent 6115 homoplasmic lines in a 4026 xanthi background. The 4026 construct expresses the plastid-targeted T7 RNA polymerase that activates E1 cellulase expression in the plastid. The major protein band at 60 kd constitutes the mature E1 cellulase. Lane 9 shows 100 ng of the *E. coli* purified catalytic form of the E1 cellulase minus the cellulose binding domain (CBD).

The *Acidothermus* E1 cellulase purified from recombinant *Streptomyces* runs as multiple forms on a denaturing gel, the highest form being 72,000 molecular weight as this form includes the endogeneous signal peptide required for secretion of the enzyme from the bacterium. The mature form of the enzyme is around 60 kilodaltons. Thus, as can be noted from Figure 3, E1 cellulase expressed in plant plastids as a 60 kd mature form and can be converted to the 40 kd catalytic domain form, presumably by proteolytic processing *in vivo*. Furthermore, from the results of the Western blot analysis it can be estimated that protein expression of E1 β-1,4-endoglucanase is approximately 1% of the total soluble plant protein in leaves of transplastomic tobacco lines.

Crude total soluble leaf protein from homoplasmic 6115 tobacco lines expressing E1 cellulase were further analyzed for cellulase activity. Since *Acidothermus* E1 cellulase V_{max} is near maximal approaching 80°C, experiments were carried out at 55°C and 80°C. Protein extracts (approximately 12 ug total leaf protein) were tested in reactions to measure the hydrolysis of the fluorogenic substrate 4-methylumbelliferyl-β-D-cellobioside (MUC) as described in Laymon *et al.* (1996) *Applied Biochem. Biotechnol.* 57/58:389-397. The results are listed in Table 1.

Table 1

Line	60min @ 55°C pM Mu/μg/min	60min @ 80°C pM Mu/μg/min	Fold Increase at 80°C
6115-7	392,063	2,432,540	6.20
6115-8	145,294	747,059	5.14
6115-10	39,706	222,549	5.60
6115-11	88,596	574,561	6.49
Xanthi (control)	n/a	13,636	n/a

The above results clearly indicate that the cellulase expressed in plant plastids has a higher level of activity at 80°C. Increases in enzyme activity of 5 to 6 fold are observed when crude extracts are incubated with MUC at 80°C than the cellulase activities obtained in incubations at 55°C. Thus, the cellulase expressed in plant plastids demonstrates similar kinetic characteristics as the wild type enzyme isolated from *Acidothermus cellulolyticus*.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

CLAIMS

What is claimed is:

1. A construct comprising as operably joined components in the 5' to 3' direction of transcription:

(a) a promoter functional in a plant cell;

(b) a DNA sequence encoding a polysaccharide hydrolyzing enzyme; and

(c) a transcription termination region.

2. The construct according to claim 1 wherein said promoter is functional in a plant plastid.

3. The construct according to claim 1 further comprising a targeting sequence capable of directing transport to a cellular organelle.

4. The construct according to claim 3 wherein said targeting sequence directs transport to a vacuole.

5. The construct according to claim 3 wherein said targeting sequence directs transport to a plastid.

6. The construct according to claim 1 wherein said enzyme is active at or above a temperature selected from the group consisting of about 45° C, about 55° C, and about 80° C.

7. The construct according to claim 1 wherein said enzyme is selected from the group consisting of cellulases, cellobiohydrolases, xylanases, ligninases and hemicellulases.

8. The construct according to claim 1 wherein said enzyme is selected from the group consisting of endocellulases and exocellulases.

9. The construct according to claim 1 wherein said DNA sequence encodes an enzyme selected from the group consisting of CBH1, xynA, lacAL, lcc1, and lccIV.

10. A plant cell containing the construct according to claim 1.

11. The plant cell according to claim 10 wherein said construct is integrated in the nuclear genome of said cell.

12. The plant cell according to claim 10 wherein said construct is integrated in the genome of plastids of said cell.

13. A plant, plant seed or plant part comprising a plant cell selected from the group consisting of the plant cell according to claim 11 and the plant cell according to claim 12.

14. A method for producing a polysaccharide hydrolyzing enzyme comprising:

growing a plant comprising a construct comprising as operably joined components in the 5' to 3' direction of transcription i) a promoter functional in a plant cell; ii) a DNA sequence encoding a polysaccharide hydrolyzing enzyme; and iii) a transcription termination region; and

isolating said enzyme from a cell of said plant.

15. A method for producing a heat optimal industrial enzyme comprising:

growing a plant comprising a construct comprising as operably joined components in the 5' to 3' direction of transcription i) a promoter functional in a plant cell; ii) a DNA sequence encoding an enzyme that degrades a cell wall component; and iii) a transcription termination region; and

isolating said enzyme from a cell of said plant.

16. A method for altering cellulose content in plant tissue comprising the steps of:

growing a plant comprising a construct comprising as operably joined components in the 5′ to 3′ direction of transcription i) a promoter functional in a plant cell; ii) a DNA sequence encoding a polysaccharide hydrolyzing enzyme; and iii) a transcription termination region, wherein said plant is grown under conditions for expression of said enzyme in a cell of said plant.

17. The method according to claim 16 wherein said enzyme degrades cellulose of said plant cell and reduces said cellulose content.

18. The method according to claim 16 wherein the digestability of plant material comprising said plant cell is improved.

19. The method according to claim 16 wherein said promoter is functional in a plant plastid.

20. The method according to claim 16 wherein said construct further comprises a targeting sequence capable of directing transport to a cellular organelle.

21. The method according to claim 20 wherein said targeting sequence directs transport to a vacuole.

22. The method according to claim 20 wherein said targeting sequence directs transport to a plastid.

23. The method according to claim 16 wherein cellulose content of plant material admixed with said plant cell is degraded.

24. The method according to claim 16 further comprising the steps of:

harvesting plant material from said plant; and

subjecting said harvested plant material to conditions wherein the activity of said enzyme is increased and cellulose content of said plant material is reduced.

25. The method according to claim 16 wherein said enzyme is active at or above a temperature selected from the group consisting of about 45° C, about 55° C, and about 80° C.

26. The method according to claim 16 wherein said enzyme is selected from the group consisting of cellulases, cellobiohydrolases, xylanases, ligninases and hemicellulases.

27. The method according to claim 16 wherein said enzyme is selected from the group consisting of endocellulases and exocellulases.

28. The method according to claim 16 wherein said DNA sequence encodes an enzyme selected from the group consisting of CBH1, xynA, lacAL, lcc1, and lccIV.

29. A method for encapsulating an enzyme that degrades a cell wall component in a plant cell organelle comprising the steps of:

growing a plant cell comprising a construct comprising as operably joined components in the 5' to 3' direction of transcription i) a promoter functional in a plant cell; ii) a DNA sequence encoding an enzyme that degrades a cell wall component; iii) a transcription termination region; and iv) a targeting sequence capable of directing transport to a cellular organelle, wherein said plant cell is grown under conditions for expression of said enzyme in an organelle of said plant cell.

30. The method according to claim 29 wherein said targeting sequence directs transport to a vacuole.

31. The method according to claim 29 wherein said targeting sequence directs transport to a plastid.

32. The method according to claim 29 wherein cellulose content of plant material admixed with said plant cell is degraded.

33. The method according to claim 29 further comprising the steps of:

harvesting plant material from said plant cell; and

subjecting said harvested plant material to conditions wherein the activity of said enzyme is increased and cellulose content of said plant material is reduced.

34. The method according to claim 29 wherein said enzyme is active at or above a temperature selected from the group consisting of about 45° C, about 55° C, and about 80° C.

35. The method according to claim 29 wherein said enzyme is selected from the group consisting of cellulases, cellobiohydrolases, xylanases, ligninases and hemicellulases.

36. The method according to claim 29 wherein said enzyme is selected from the group consisting of endocellulases and exocellulases.

37. The method according to claim 29 wherein said DNA sequence encodes an enzyme selected from the group consisting of CBH1, xynA, lacAL, lcc1, and lccIV.

Plasmid Map of pCGN6115

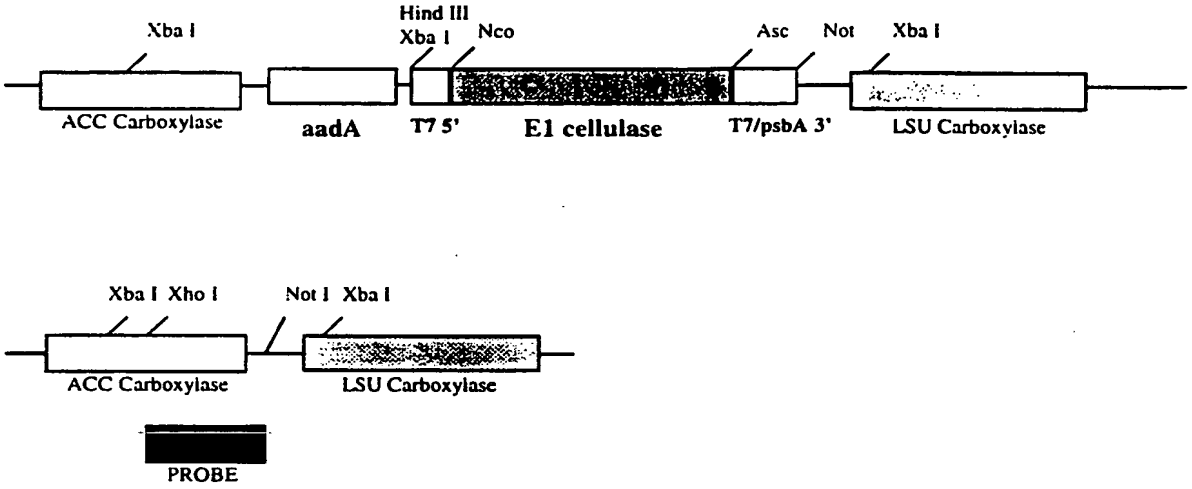

Figure 1

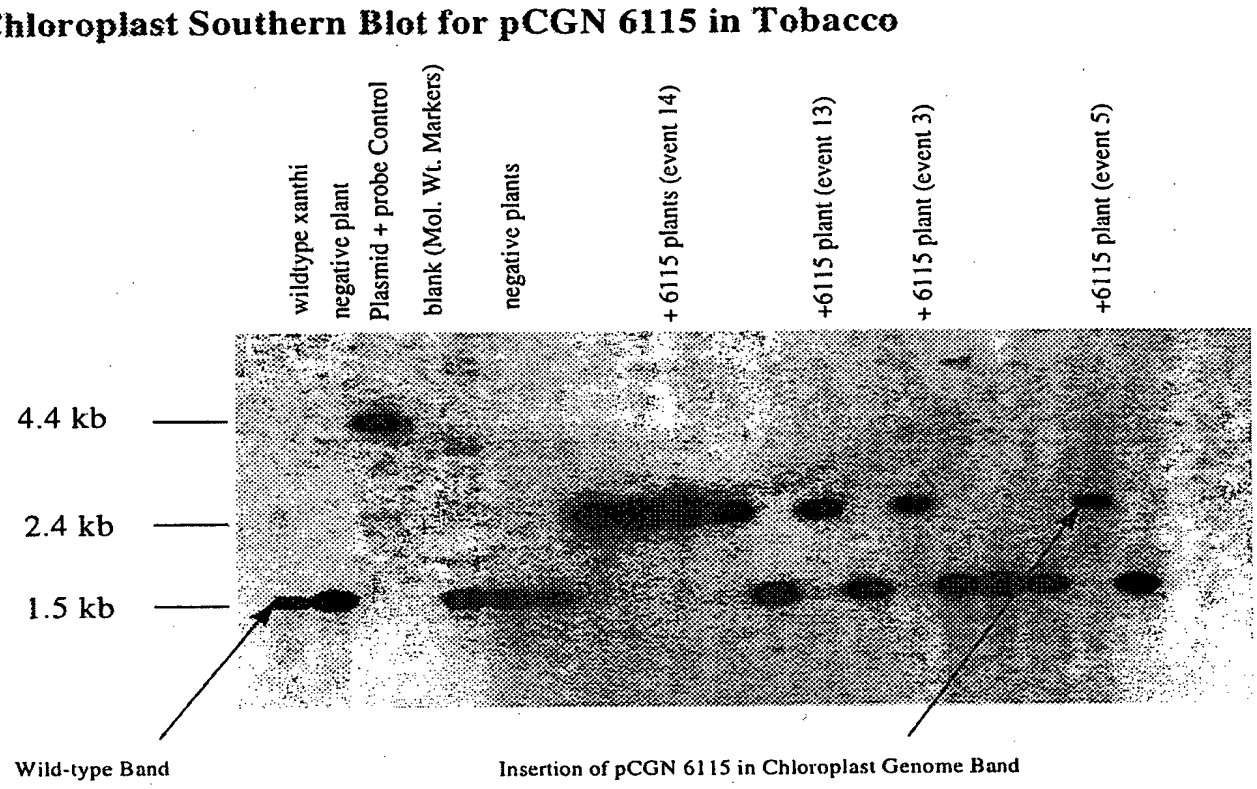

Figure 2

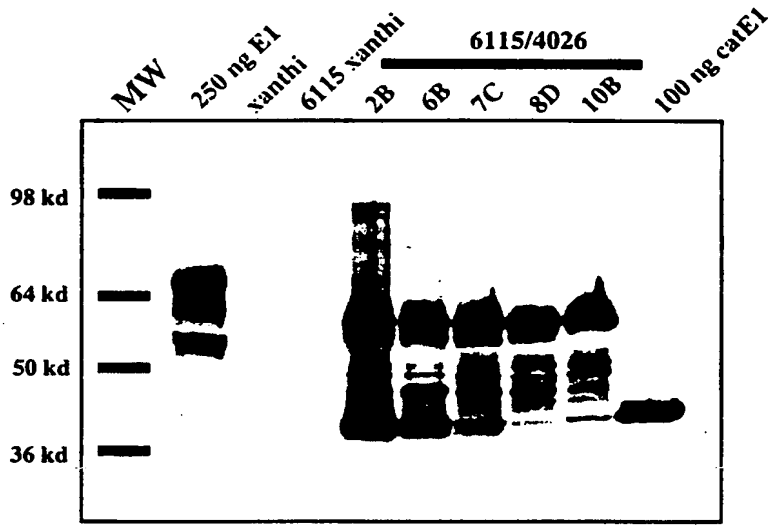

Figure 3

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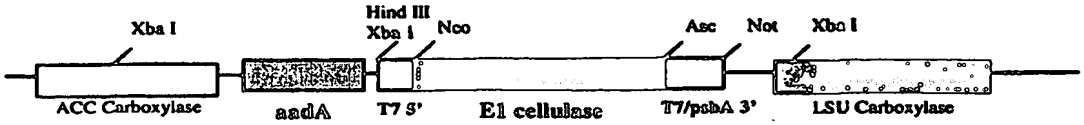
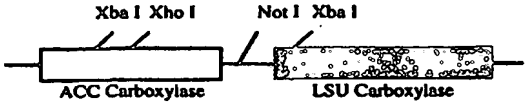
Plasmid Map of pCGN6115





(57) Abstract

Novel compositions and methods useful for genetic engineering of plant cells to provide expression of cellulose degrading enzymes in the plants or plant cells.

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CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/16579

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N15/55 C12N15/82 C12N5/10 A01H5/00
A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HERBERS K. ET AL.: "Apoplastic expression of the xylanase and beta (1-3,1-4) glucanase domains of the xynD gene from Ruminococcus flavefaciens leads to functional polypeptides in transgenic tobacco plants" MOLECULAR BREEDING, vol. 2, 1996, pages 81-87, XP002130591 the whole document ---	1,7,10, 11,13,14
X	LIU J. ET AL.: "Plant seed oil-bodies as an immobilization matrix for a recombinant xylanase from the rumen fungus Neocallimastix patriciarum" MOLECULAR BREEDING, vol. 3, 1997, pages 463-470, XP002130592 the whole document ---	1,3,7, 10,11, 13,14, 29,32, 33,35

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 February 2000	06.03.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 190 194 A (MOLONEY MAURICE M ;LIU JIN HAO (CA); CHENG KUO JOAN (CA); FORSBERG) 13 May 1998 (1998-05-13) see esp. example C	1,3,7, 10,11, 13,14, 29,32, 33,35
X	HERBERS K ET AL: "A THERMOSTABLE XYLANASE FROM CLOSTRIDIUM THERMOCELLUM EXPRESSED AT HIGH LEVELS IN THE APOPLAST OF TRANSGENIC TOBACCO HAS NO DETRIMENTAL EFFECTS AND IS EASILY PURIFIED" BIO/TECHNOLOGY,US,NATURE PUBLISHING CO. NEW YORK, vol. 13, no. 1, 1 January 1995 (1995-01-01), pages 63-66, XP002008876 ISSN: 0733-222X	1,6,7, 10,11, 13-18, 23-26
Y	the whole document	2-5,9, 12, 19-23, 28-35,37
X	WO 97 45549 A (CENTRE NAT RECH SCIENT ;FAYE LOIC (FR); GOMORD VERONIQUE MARTINE () 4 December 1997 (1997-12-04)	1,7,10, 11,13, 14, 16-18, 24,26
Y	the whole document	2-5,12, 19-23, 29-33,35
X	WO 98 11235 A (CIBA GEIGY AG ;HEIFETZ PETER (US); LEBEL EDOUARD (US); UKNES SCOTT) 19 March 1998 (1998-03-19)	1-37
Y	see the whole document; esp. examples B, C	2-5,9, 19-22, 28-37
X	WO 98 16651 A (WISCONSIN ALUMNI RES FOUND) 23 April 1998 (1998-04-23)	1,6-8, 10-18, 23-27
Y	the whole document	2-5,9, 19-22, 28-37
X	OHMIYA, KUNIO ET AL: "Relaxation of biomass tissue by expressing bacterial genes encoding xylanases and cellulases" BIOTECHNOL. SUSTAINABLE UTIL. BIOL. RESOUR. TROP. (1997), 11, 24-33, XP002118613 see the whole document; esp. pp.28-31	1-3,6-8, 10,11, 13-19, 23,24,27

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 02551 A (MIDWEST RESEARCH INST) 1 February 1996 (1996-02-01) cited in the application the whole document ---	1-37
A	SHOEMAKER ET AL.: BIOTECHNOLOGY, vol. 1, 1983, pages 691-696, XP000653365 cited in the application the whole document -----	9,28,37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/16579

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7,9-26,28-35,37 partially; 8,27,36 completely

A construct comprising a promoter functional in a plant cell, a DNA sequence encoding a polysaccharide hydrolyzing enzyme and a transcription termination region. Said construct optionally comprising: a promoter being functional in a plant plastid, a targeting sequence for directing transport to a cellular organelle, preferably to vacuoles and plastids. Said enzyme optionally being active at or above temperatures of 45AC, 55AC, 80AC, being a cellulase (endo- or exo-) or cellobiohydrolase, preferably being a CBH1 enzyme. Plant cells, plants, and seeds comprising said constructs.
Use of said constructs in methods for: the production of polysaccharide hydrolyzing enzymes, preferably heat optimal industrial enzymes degrading cell walls in plants, methods for altering cellulose content in plant tissue, methods for encapsulating an enzyme that degrades a cell wall component in a plant cell organelle.

2. Claims: 1-7,9-26,28-35,37 partially

idem, the enzyme being a hemicellulase, preferably a xylanase, more preferably a xynA enzyme

3. Claims: 1-7,9-26,28-35,37 partially

idem, the enzyme being a ligninase or laccase, preferably being selected from the group of lacAL, lcc1, and lccIV.

INTERNATIONAL SEARCH REPORT

Information on patent family members

ational Application No
PCT/US 99/16579

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA 2190194 A	13-05-1998	NONE	
WO 9745549 A	04-12-1997	FR 2749322 A	05-12-1997
		AU 3097297 A	05-01-1998
WO 9811235 A	19-03-1998	AU 4414697 A	02-04-1998
		CN 1230224 A	29-09-1999
		EP 0925362 A	30-06-1999
		PL 331767 A	02-08-1999
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		AU 4980797 A	11-05-1998
		BR 9712538 A	19-10-1999
		EP 0932692 A	04-08-1999
WO 9602551 A	01-02-1996	US 5536655 A	16-07-1996
		AU 682125 B	18-09-1997
		AU 3098595 A	16-02-1996
		CA 2194478 A	01-02-1996
		EP 0771321 A	07-05-1997
		US 5712142 A	27-01-1998